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(54) Title: ENHANCED DELIVERY OF NUCLEIC ACID–BASED DRUGS

(57) Abstract

The invention provides methods and associated compositions for enhancing the intracellular delivery of a nucleic acid–based drug, such as an oligonucleotide or gene, by administering the nucleic acid–based drug in combination or sequentially with an enhancer. Preferred enhancers are caprylic acid, capric acid or their pharmaceutically acceptable salts. The preferred route of administration is oral.

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ENHANCED DELIVERY OF NUCLEIC ACID-BASED DRUGS

FIELD OF THE INVENTION

The present invention relates to the use of permeation enhancers, particularly paracellular and transcellular permeation enhancers, to improve the intracellular delivery to mammalian cells of nucleic acid-based drugs such as antisense oligonucleotides and genes. In particular, this invention relates to formulations which contain a nucleic acid-based drug in combination with at least one permeation enhancer, such as caprylic acid, capric acid or their pharmaceutically acceptable salts, to facilitate the intracellular delivery of the nucleic acid-based drug.

BACKGROUND OF THE INVENTION

Several issues are associated with the delivery of nucleic acid-based drugs, such as antisense oligonucleotides and genes, into cells. Cellular delivery of oligonucleotides has been a challenge and it has been reported that only 1-2% of oligonucleotides are taken up by cells (1988, *Gene* 72, 333-341; 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86, 3474-3478). Cell penetration, release from endosomal compartments and intracellular distribution of antisense oligonucleotides are among the major limiting factors for the effective use of antisense oligonucleotides as drugs. Because cell membranes are negatively charged, the passive diffusion of phosphorothioate-based antisense oligonucleotides across the lipid bilayer is a major challenge. Furthermore, oligonucleotide length, chemistry ,

concentration and sequence have been shown to be determinants of cellular uptake. Cell type, stage of cell cycle, degree of cell differentiation have also been proposed to affect uptake levels (1998, *J. Drug Targeting* 5, 225-234; 1995, *J. Pharmacal. Toxicol.* 275, 462).

Several mechanisms by which oligonucleotides might be internalised have been identified. The internalisation process is time and temperature dependent and saturable, suggesting that an active/energy-dependent mechanism is involved. Receptor-mediated endocytosis of oligonucleotide sequences has been observed in a number of cell types. For instance, proteins of 80 and 30kDa have been identified that mediate oligonucleotide uptake, whereas a simple charge association with the membrane has been found to be sufficient to trigger endo- or pinocytosis (1989, *Proc. Natl. Acad. Sci. U.S.A.* 86, 6454-6458; 1985, *J. Clin. Invest.* 76, 2182-2190).

Once inside the cell, oligonucleotides have been shown to partition to various regions of the cell, depending on their chemistry. For example phosphodiester- and methylphosphonate-based oligonucleotides are sequestered within the nucleus in most of the cell types examined (1990, *New Biol.* 9, 1091-1100). Phosphorothioate derivatives remain within the cytoplasm in some cell lines, but enter the nucleus in others (1996, *Trends Biotechnology* 14(5), 147-9).

WO99/01579 discloses that certain permeation enhancers are able to increase uptake of nucleic acids from the alimentary canal into the bloodstream.

Despite these efforts and others, the intracellular delivery, especially via the oral route, of pharmacologically effective amounts of nucleic acid-based drugs remains problematic.

SUMMARY OF THE INVENTION

It has now been discovered that enhancers such as sodium caprylate and sodium caprate can be used to achieve elevated intracellular delivery of a nucleic acid-based drug to mammalian cells. In particular, the enhancers are capable of increasing delivery of nucleic acid-based drugs to the cytoplasm and/or nucleus of cells, compared to the delivery to these regions in the absence of enhancer. In the absence of enhancer, uptake of nucleic acid, if any, tends to be weak and into intracellular vesicles such as endosomes rather than into the cytoplasm or nucleus.

The enhanced delivery of the invention can lead to increased biological activity *in vitro* and *in vivo*, such as substantially decreased expression of a particular receptor upon administration in the presence of an enhancer of an antisense oligonucleotide to that receptor or increased efficiency of DNA transfection upon delivery of plasmid DNA, condensed plasmid DNA or plasmid DNA associated with cationic lipid(s) or cationic polymer(s) in combination with an enhancer in comparison to DNA delivered in the absence of the enhancer.

Thus, this invention provides methods and associated uses and compositions for enhancing the intracellular delivery of a nucleic acid-based drug, such as an oligonucleotide

or gene, that comprise the administration of the nucleic acid-based drug in combination or sequentially with an enhancer. The enhancer is present in an amount effective to enhance the intracellular delivery of the nucleic acid-based drug. Preferred enhancers include caprylic acid, capric acid and their pharmaceutically acceptable salts.

Elevated intracellular delivery may be indicated by homogeneous, rather than punctate, distribution of the nucleic acid-based drug within the cell, particularly within the cytoplasm and/or nucleus, or by measuring activity of the drug, for example elevated expression of a protein encoded by the drug, or reduced expression in the case of an antisense drug. The distribution or activity may be determined by any convenient manner, such as those exemplified herein, or others well known to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a shows the cell survival (% relative to untreated cells) for Caco-2 cells incubated for either 30 min or 150 min with C8 at concentrations of 120, 100, 80, 60,40, 20 and 10 mM using the MTT assay as described in Example 1, Experiment d. **Fig. 1b** shows the cell survival (% relative to untreated cells) for Caco-2 cell incubated for either 30 min or 150 min with C10 at concentrations of 14, 12, 10, 8, 6, 4, and 2 mM using the MTT assay as described in Example 1, Experiment d;

Fig. 2 shows a Western blot analysis depicting the expression of HPT-1 receptor in Caco-2 non-differentiated

cells after treatment with antisense oligonucleotides in combination with C8 enhancer according to Example 2. **Panel A** shows the treatment with S-PTH-01 antisense oligonucleotide while **Panel B** shows the treatment with S-PTH-02 antisense oligonucleotide. The concentrations of both antisense and enhancers used is indicated. Incubation time was 1 hr for all the samples unless indicated otherwise;

Fig. 3 shows, as further described in Example 3, the expression of rat PT-1 receptor in intestinal samples of rat treated with antisense oligonucleotide, 25mg C10 or a mix of both. Animals were allowed to recover for 24hrs after treatment;

Fig. 4 shows, as further described in Example 3, the expression of rat PT-1 receptor in intestinal samples of rat treated with antisense oligonucleotide and 5 or 25mg C10 enhancer. Animals were allowed to recover for 48hrs after treatment; and

Fig. 5 shows a comparison of transfection efficiencies for β-galactosidase in Caco-2 cells as described in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

It has now been discovered that permeation enhancers such as C8 and C10 (see later descriptions) can be used to achieve elevated intracellular delivery of antisense oligonucleotides and other nucleic acid-based drugs to the cytoplasm and/or nucleus and other intracellular regions or compartments of epithelial cells, as required

for their pharmacological activity. This enhancement in intracellular delivery is unlikely to be restricted to epithelial cells and most likely can be applied to other or all cell types and tissue types. Furthermore, it has been discovered that this enhanced delivery of antisense oligonucleotides upon co-administration with an enhancer can lead to increased biological activity *in vitro* and *in vivo*. For instance, administration of an antisense oligonucleotide to a particular receptor in combination with an enhancer can lead to substantially decreased expression of that receptor in cell models and *in vivo* such as in the gastrointestinal tract.

It has further been discovered that enhancers, such as C8 and C10, can also facilitate the intracellular delivery of plasmid DNAs, such as plasmid DNA complexed with a model cationic lipid such as lipofectamine, into Caco-2 cells. For instance, it has been discovered that certain enhancers increase the efficiency of DNA transfection in comparison to DNA complexed with cationic lipids alone. Condensed plasmid DNA complexed with cationic lipids can also be used with the enhancer in order to achieve enhanced intracellular gene delivery as manifested by increased gene expression.

Because of this enhanced intracellular delivery of the oligonucleotides and plasmid DNAs in the presence of various enhancers such as C8 and C10 in epithelial cell models, formulations or drug dosage forms containing these combinations can be used in order to achieve local delivery of nucleic acid-based drugs, such as oligonucleotides or plasmid DNAs, at different sites in the body (be it human or other mammals) including the

gastro-intestinal tract, sub-dermal sites, intra-muscular sites, or indeed any sites in the body (human or other mammals) in which both the nucleic acid-based drug and the enhancers can be delivered either together or in sequential fashion. Thus, the nucleic acid-based drug can be administered first followed by administration of the enhancer or the enhancer can be administered first followed by administration of the nucleic acid-based drug. Example routes of administration are oral, parenteral, nasal, inhalation, vaginal, rectal, intradermal and topical. As such, this invention is useful for treating a mammal that can benefit by administration of a pharmacologically effective amount of a nucleic acid-based drug, such as to prevent or treat a condition prevented or ameliorated by the drug.

Furthermore given the ability of enhancers to elevate intracellular delivery of oligonucleotides and plasmid DNAs (either single stranded or double stranded) to mammalian cells, it is proposed that when the enhancers are administered with other nucleic acid-based drugs, such as ribozymes, gene-correcting oligonucleotides, triple-helix forming oligonucleotides, oligonucleotides which function as adjuvants (such as oligonucleotides containing CpG motifs), these nucleic acid-based drug/enhancer combinations will also achieve the improved intracellular delivery of the nucleic acid-based drug into mammalian cells and into tissues.

Additionally, the discovery that enhancers facilitate the intracellular delivery of plasmid DNAs has broad application to the intracellular delivery of plasmid DNAs and genes to mammalian cells using non-viral vector

systems including, but not limited to, cationic lipids, PEI (polyethyleneimine) systems, polyanhydride systems, chitosan systems, cellulose systems, dendrimeric based systems, liposomes, PLGA DNA particles co-administered with enhancers. The nucleic acid-based drug can be complexed, for example by covalent **or** non-covalent bonding, or entrapped by these polymeric systems or can be incorporated as a condensed plasmid complex into such non-viral vector systems using, for example, protamine phosphate or protamine sulphate as the condensing agent.

Additionally, further enhancement of intracellular delivery of a nucleic acid-based drug can be obtained by co-administration of the nucleic acid-based drug/enhancer with an inhibitor of an enzyme that degrades the nucleic acid-based drug, or which expels or effluxes the nucleic acid-based drug from the cell. Example inhibitors of an enzyme that degrades a nucleic acid-based drug include P-glycoprotein inhibitors, such as verapamil, ketaconazole, diltiazem and the like. The nucleic acid-based drug/enhancer combination can also be co-administered with an endosome escape and/or nuclear accumulation agent to further control the location of the nucleic acid-based drug within the cell. Example endosome escape/nuclear accumulation agents include agents having a REDL endosomal escape motif or a KKKRKA nuclear localisation motif. Such agents can be conjugated to agents such as farnesyl, AFCME or myristoyl motifs in order to increase lipophilicity. Additionally, the nucleic acid-based drug can be condensed by a DNA condensing agent, such as poly-lysine, protamine or calcium phosphate, and administered in the presence of an enhancer.

Furthermore, combinations of oligonucleotides with enhancer type systems may also afford improved intracellular delivery of oligonucleotides into prokaryotic cells including *Escherichia coli, Helicobacter pylori, Salmonella typhiminum* and other infectious prokaryotic agents and in doing so facilitate the delivery of nucleic acid-based drugs including oligonucleotides to such infectious agents in order to arrest their growth rate or kill them.

The "enhancer" is preferably a fatty acid or an ester, an ether, a salt or a derivative thereof, such as medium chain and long chain fatty acids and their corresponding mono-, di- and triglycerides, which is, preferably, solid at room temperature and which, preferably, has a carbon chain length of from 4 to 24 carbon atoms; with the provisos that (i) where the enhancer is an ester of a fatty acid, said chain length of from 4 to 24 carbon atoms relates to the chain length of the carboxylate moiety, and (ii) where the enhancer is an ether of a fatty acid, at least one alkoxy group has a carbon chain length of from 4 to 24 carbon atoms. An "enhancer" also encompasses a combination of one or more enhancers (including physical and covalent/conjugate combination). As used herein, a "derivative" of a fatty acid, or of an ester, ether or salt thereof, means a pharmaceutically acceptable agent which contains a straight chain component derived from a fatty acid, such as a hydrocarbon chain containing 8 or 10 carbons, that is effective in enhancing the uptake of a nucleic acid-based drug in mammalian cells or tissue. Enhancers can be cationic or anionic in nature.

Examples of enhancers include, but are not limited to, caprylic acid, nonanoic acid, capric acid, undecanoic acid, lauric acid, acylcarnitines such as palmitoyl carnitine, babassu oil, fish oils such as eicosapentaenoic acid or docosahexaenoic acid, and medium chain glyceride mixes.

Preferably, the enhancer is a medium chain fatty acid, or an ester, an ether, a salt or a derivative of a medium chain fatty acid which is, preferably, solid at room temperature and which has a carbon chain length of from 8 to 14 carbon atoms; with the provisos that (i) where the enhancer is an ester of a medium chain fatty acid, said chain length of from 8 to 14 carbon atoms relates to the chain length of the carboxylate moiety, and (ii) where the enhancer is an ether of a medium chain fatty acid, at least one alkoxy group has a carbon chain length of from 8 to 14 carbon atoms. Preferred medium chain fatty acids for use as enhancers (per se or in the form of an ester, an ether, a salt or a derivative) are caprylic, nonanoic, capric, undecanoic and lauric acids. More preferred are caprylic and capric acids.

More preferably, the enhancer is a pharmaceutically acceptable salt, preferably the sodium salt, of a medium chain fatty acid, the medium chain fatty acid having a carbon chain length of from 8 to 14 carbon atoms.

Even more preferably, the enhancer is sodium caprylate, sodium caprate or sodium laurate. As used herein, the terms "C8" and "C10" refer to sodium caprylate and sodium caprate, respectively, and "nonanoate" and "undecanoate" refer to sodium salts.

As used herein, the term "nucleic acid-based drug" means an oligonucleotide, an antisense oligonucleotide including oligonucleotides having a modified backbone chemistry such as phosphorothioate, phosphotriester, methylphosphonate or phosphoramidate oligonucleotides, oligonucleotides having modified sugars or terminal groups such as 2' methoxy substitution, poly-lysine terminally modified oligonucleotides or 2'-O-alkyl-oligoribonucleotides, chimeric oligonucleotides comprised of nucleotides of different chemistries, oligonucleotides having MOE chemistries or other known chemistries shown to function as antisense oligonucleotides, ribozymes, gene-correcting oligonucleotides, triple-helix forming oligonucleotides, oligonucleotides which function as adjuvants, plasmid DNAs, and genes. In addition a nucleic acid-based drug includes a gene coding for a protein of interest such as a therapeutic protein, a prophylactic or therapeutic vaccine or a cancer chemotherapeutic; a gene coding for an RNA molecule which functions in an antisense capacity when expressed within mammalian cells and a gene coding for a ribozyme.

The amount of enhancer effective to enhance the intracellular delivery of a nucleic acid-based drug ranges from about 0.005mM to about 1M, preferably from about 0.01mM to about 100mM. The optimum concentration for a particular enhancer depends upon a number of factors such as the size of the oligonucleotide to be delivered and the chain length of the enhancer. Particularly preferred concentrations are 1 to 200mM, more preferably 5 to 100mM, for capric acid-derived enhancers and 0.5 to 20mM, more preferably 1 to 10mM for caprylic acid-derived enhancers. Typically, the molar

ratio of the enhancer to the nucleic acid-based drug can range from about 1:100 to about 100:1.

Suitable pharmaceutical compositions include liquid, semi-solid or solid dosage forms, such as solutions, suspensions, emulsions, microemulsions, capsules, tablets and microparticulates.

Example 1 - Uptake of antisense phosphorothioate oligonucleotides in non-differentiated Caco-2 cells in the presence of permeation enhancers.

Caco-2 cells were seeded on coverslips treated with polylysine at a concentration of approximately 2.5 - $5x10^5$ cells/ml and grown to confluence. Cells were washed once with PBS without Ca^{2+}, Mg^{2+}. 1-2 ml of PBS w/o Ca^{2+}, Mg^{2+} containing antisense oligonucleotides +/- enhancers was added and cells were incubated for 30 min at 37°C. Variations to this protocol by pre-incubating cells with enhancers (40min), washing with PBS w/o Ca^{2+}, Mg^{2+} and then incubating with antisense oligonucleotides for 30min were also analysed.

Two different FITC labelled antisense oligonucleotides were used: FITC-labelled ELNG05 (5'-GCCCACCGGGTCCACCAT-3'; complementary to a sequence in the human BCR-Abl mRNA) and FITC-labelled S-PTH-05 (5'-CTAAACTTCCCCTCTTGG-3', complementary to sequence 153-170 of the human HPT1 receptor mRNA). The enhancers were used at a range of concentrations: C8 from 120mM to 0.12mM and C10 from 13mM to 0.013mM. The uptake of antisense oligonucleotides was also analysed in the absence of enhancers for variable periods of time (30min - 60min). Following incubation,

cells were washed twice with PBS w/o Ca^{2+}, Mg^{2+} and stained with 1μg/ml TexasRed-DHPE (dihexadecanoylglycerophospho-ethanolamine, Molecular Probe T-1395) for 15min at 37°C, washed extensively and fixed with paraformaldeyde pH shift (Barcallo, 1995) and mounted for analysis by confocal laser scanning microscopy (CLSM).

Variations of this general protocol and results obtained from these experiments are given below.

Experiment a

Caco-2 cells were incubated with 2ml of enhancers in PBS w/o Ca^{2+} and Mg^{2+} (C8: 80mM and 120mM; C10: 10mM and 13mM) and incubated for 40min at 37°C. After two washes with PBS w/o Ca^{2+} and Mg^{2+}, the cells were incubated with 2ml of FITC-antisense oligonucleotide (ELNG05) at a concentration of 10μM in PBS w/o Ca^{2+} and Mg^{2+} and incubated at 37°C for 30min.

Analysis by CLSM indicated that very limited antisense uptake occurred in the absence of any enhancers and that the intracellular distribution was in vesicular compartments. Addition of C8 at 80-120mM and C10 at 10-13mM permeated completely the cells causing intracellular uniform staining with TexasRed-DHPE and FITC-oligonucleotide.

Experiment b

Caco-2 cells were treated with a PBS w/o Ca^{2+} and Mg^{2+} solution containing both an antisense oligonucleotide and an enhancer (10μM antisense; either S-PTH-05 or ELNG05,

C8: 120, 24, 12, 2.4, 1.2, 0.24, 0.12mM; C10: 13, 2.6, 1.3, 0.26, 0.13, 0.026, 0.013mM) and incubated for 30min at 37°C.

CLSM analysis does not show any cytoplasmic or nuclear accumulation of antisense in the absence of enhancers but granular distribution of FITC-staining seems to be present both on the surface and inside the cells consistent with uptake into vesicles but not into nucleus or cytoplasm. Addition of C8 at 120mM confirmed the permeation of the cells and, interestingly, both FITC-oligonucleotide and TexasRed DHPE accumulated in the nucleus. When decreasing the concentration of C8 to 24mM, some cells showed cytoplasmic or nuclear accumulation of FITC-antisense but the majority had a pattern similar to the control. Lower C8 concentrations were identical to the control. The higher concentration of C10 (13mM) caused a loss of adherence of Caco-2 cells in both the experiments. The 2.6 mM C10 concentration did not increase the uptake of antisense.

Experiment c

Caco-2 cells were incubated with 1ml of antisense +/- enhancers in PBS w/o Ca^{2+} and Mg^{2+} and incubated for 30min at 37°C. Two different fluorescently labelled antisense oligonucleotides were used in these experiments at a concentration of 10μM: ELNG05 (antisense to BCR-Ab1) and S-PTH-05 (antisense to HPT1 receptor). C8 enhancer was used at 120, 100, 80, 60, 40 and 20 mM; C10 enhancer was used at 13, 10, 8, 6, 4 and 2 mM. The uptake of antisense was also analysed in the absence of enhancers for 30min and 60min as control.

In absence of enhancers, granular distribution of the fluorescent-staining was observed; antisense oligonucleotides seem to be present on the surface of the cells and possibly in the cytoplasm after 30min of incubation for both ELNGO5 and SPTH-FITC05. With longer incubation (60min), a slight increase of intracellular antisense could be observed.

Using C8 enhancer at a concentration of 120mM, a complete staining of cells with both the fluorescence oligonucleotides used was observed, indicating that cells were completely permeabilized. When the concentration was decreased to 100mM and 60mM, a nuclear accumulation of FITC-antisense was observed. Similar results have been obtained with both the antisense analysed. By decreasing the concentration of C8 further (i.e. 40mM) a decrease uptake was observed and only a few cells show the presence of antisense in vacuoles and inside the plasma membrane.

C10 enhancer caused the destruction of cells at concentrations between 13mM and 8mM. Only a few cells remained attached to the coverslip during the experimental manipulation. At a concentration of 10mM the cells were completely stained, indicating permeabilization of the plasma membrane. At a concentration of 8mM only punctate staining, similar to the control, could be observed.

As shown above, medium chain fatty acid surfactants such as C8 and C10 increase the Caco-2 plasma membrane permeability and promote the uptake of oligonucleotides. Although the highest concentrations of enhancers used in

the experiments may cause mortality of cells in culture, but not necessarily cells in tissues, concentrations below the toxicity values are still able to increase the cellular uptake and/or accumulation into the nucleus. Furthermore, prolonged incubation of mammalian cells or tissue with the oligonucleotides and enhancers may further result in enhanced intracellular delivery of the oligonucleotides to mammalian cells or tissues.

Experiment d - Cytotoxicity of Enhancers on Caco-2 cells

Caco-2 cells were seeded at 10^5 cells/well in 96-well plates and grown overnight at 37°C. An enhancer at a particular concentration was added to the cells (10µl per well) and cells were incubated for 30min or 150min. 10µl of the MTT labeling reagent from the colorimetric MTT based cell proliferation kit (Boehringer #1465007) was added to each well and cells were incubated for 4 hr at 37°C. 100µl of solubilization buffer was added to each well, cells were incubated overnight and absorbance was read at 570nm. Fig. 1 shows the results of these cytotoxicity studies for C8 (Fig. 1a) and C10 (Fig. 1b) expressed as cell survival (% of untreated cells) for C8 concentrations of 10 to 120 mM and for C10 concentration of 2 to 14 mM.

The above experiments show that the enhancers C8 and C10 dramatically increase the intracellular delivery of FITC-labelled oligonucleotides of the phosphorothioate chemistry. This enhancement in intracellular delivery of the antisense oligonucleotides in the presence of the enhancers C8 and C10 is very rapid, being detectable after 30 minutes in Caco-2 cells. Furthermore, this

enhancement in the intracellular delivery of the oligonucleotides is achievable at concentrations of enhancers which are not toxic to Caco-2 cells. From the confocal microscopy studies it is evident that, in the presence of the enhancers, the FITC-labeled oligonucleotides are distributed through-out the cell interior including cytoplasmic and nuclear localisation and are not restricted to punctate distribution which may have been attributed to vesicular or endosomal localisation. More prolonged incubation of mammalian cells with the oligonucleotide/enhancer combination may further enhance the cellular uptake and intracellular distribution to both the cytoplasm and the nucleus. Experiments similar to those described above undertaken with polarised Caco-2 cells gave similar results.

Example 2 - The effect of antisense oligonucleotides to the HPT1 receptor on the expression of HT-1 in non-differentiated Caco-2 cells: Permeation enhancers promote uptake and biological function of phosphorothioate oligonucleotides.

Caco-2 cells were grown in a 6 well plate until 70-80% confluent. Cells were treated with antisense oligonucleotides S-PTH-O1 (5'-GGGCCTGAAGTATCATAG3'; nucleotides 86-103 of human HPT1 sequence) and S-PTH-02 (5'-CTAAACTTCCCCTCTTGG-3'; nucleotides 153-170 of human HPT1 sequence) at concentrations of 1 or 10 μM in 1ml PBS w/o Ca^{2+} and Mg^{2+}. Cellular permeation enhancers C8 and C 10 were added to the antisense mix at concentrations of 40mM, 60mM for C8 and 6mM, 10mM for C10. Caco-2 control wells received either antisense or enhancers only. Untreated cells were also included. Cells were incubated

for 30 min or 2h (only a few samples were treated for 2h: 10μM antisense plus 40mM C8 or 6mM C 10), washed twice with PBS and fresh medium was added. Caco-2 cells were incubated for 36h at 37°C.

After incubation, cells were washed twice with PBS and recovered in buffer containing protease inhibitors (0.1M NaCl, 0.01M TrisHCl pH7.6, 1mM EDTA pH8.0, Boehringer Protease Inhibitor cocktail). Samples with floating cells were collected, centrifuged, washed with PBS and resuspended in the same buffer. Cell suspensions were frozen at -80°C. Samples were defrosted, homogenised (hand-homogeniser) and protein concentration quantified by Bradford assay using the Bio-Rad kit. Equal amounts of protein (10μg of total cell lysate) were loaded onto a 10% SDS-polyacrylamide gel for electrophoresis. Once samples were transferred to nitrocellulose membranes, the membranes were subjected to standard Western blot blocking and screening treatments with staining for 1 h with rabbit anti-HPT1 polyclonal antibody (dil. 1:1000) followed by incubation with secondary antibody conjugated to horseradish peroxidase (goat anti rabbit IgG-HRP, Sigma A0545, dil. 1:5000). The results were visualised by enhanced chemiluminescence detection using standard chemiluminescence treatment.

Fig. 2 shows the expression of HPT-1 receptor in Caco-2 non-differentiated cells after treatment with antisense oligonucleotides in combination with C8 enhancer. The treatment with two different antisense oligonucleotides to HPT-1 is indicated. The expression of HPT-1 receptor in non-differentiated Caco-2 cells is substantially

decreased after 2hrs incubation with a mix of antisense oligonucleotides and 40mM C8 enhancer.

It is also noteworthy that treatment of the cells with 1μM antisense S-PTH-01 and 40mM C8 for 60 min also resulted in decreased HPT1 expression as determined by Western Blot studies relative to the control, untreated cells (Fig. 2). In addition, treatment of cells with 10μM antisense S-PTH-01 and 60mM C8 for 60 minutes also decreased the expression of HPT1 relative to the control, untreated cells (Fig. 2). Furthermore, treatment of cells with 1 or 10μM S-PTH-02 and 60 mM C8 also decreased the expression of HPT1 relative to untreated cells and relative to cells that were treated with 1μM of this oligonucleotide and 40mM C8 (Fig. 2).

These results indicate that the expression of HPT-1 receptor in non-differentiated Caco-2 cells is substantially decreased after 2hrs incubation with a mix of antisense oligonucleotides and 40mM C8 enhancer.

Furthermore, these results indicate that expression of HPT-1 receptor is decreased in these cells treated with either the S-PTH-01 or S-PTH-02 oligonucleotide together with 40mM or 60mM C8 for 60 minutes relative to control, untreated cells. Thus, a decrease in HPT-1 expression could be argued for lower incubation time (1 hr) for both the concentrations of antisense oligonucleotides used (1 and 10μM).

In Example 1 we demonstrated that treatment of Caco-2 cells with a FITC-labelled oligonucleotide together with either C8 or C 10 results in enhanced intracellular

uptake and delivery of the FITC-labelled oligonucleotide to the cells relative to control cells treated with the oligonucleotide alone as determined by confocal microscopy. The studies reported here further demonstrate that treatment of cells with the antisense oligonucleotides S-PTH-01 or S-PTH-02 together with the enhancer C8 at either 40mM or 80mM concentrations for either 60 min or 2 hours results in the down-regulation in expression of the HPT1 receptor as demonstrated by Western Blot studies. Thus we have demonstrated that administration of antisense oligonucleotides to Caco-2 cells together with penetration enhancers increases the intracellular uptake and delivery of the antisense molecules and results in down-regulation of target protein expression. As such, we have demonstrated that co-administration of oligonucleotides together with penetration enhancers increases cellular uptake of same and that such molecules retain activity once delivered to the cells. This is a general way to deliver oligonucleotides to cells and may have application for the delivery of nucleic acids in general to mammalian cells or across biological barriers. In addition, initial treatment of mammalian cells with the enhancer followed by subsequent treatment with the antisense (and vice versa) should also increase the intracellular uptake into mammalian cells and tissue.

Example 3 - Effect of antisense oligonucleotide to HPT-1 receptor on HPT-1 expression in vivo: The cellular permeation enhancer capric acid (C10) promotes the intracellular uptake and biological function of a phosphorothioate antisense oligonucleotide when delivered in the rat closed loop model.

Wistar rats in the 190-250 g weight range were fasted for 24 hours prior to study initiation. Animals were anaesthetised with sagetal prior to dose administration and during the dosing procedure were given halothane gas. Injections of drug were made directly into the intestine closed loop 12cm below the pyloris and after four hours the sutures were removed from the intestine. The animals were allowed to recover (food and water available) for 24 or 48 hours. At the termination of the experiment the animals were sacrificed and tissue samples from the intestine (site of injection), liver, kidney, spleen, lung and heart were taken, frozen in dry ice and stored at -80°C. Intra-duodenal injection of antisense oligonucleotide alone, C10 enhancer alone or a mixture of antisense oligonucleotide and enhancer were administrated to two groups of animals (N=4 per group) and the rats were allowed to recover for either 24 hour or 48 hours. Antisense oligonucleotide S-PTH-03 (5'-CCACTATAGCTCCCTGAG-3'; antisense to rat PT-1 receptor, nucleotides 119-136) was administered at concentration of 10μM, C10 enhancer was used at 5mg or 25mg in combination with antisense and at 25mg for a control group.

Intestinal samples were defrosted on ice in PBS buffer with protease inhibitors (Boehringer), the intestine was cut with a scalpel and rat intestinal epithelial tissue was prepared by mucosal scraping using a glass slide. The samples were homogenised in a sucrose buffer containing protease inhibitors and the homogenates were centrifuged at 4000 RPM for 15 minutes. 100μg of total protein from each sample were run on 10% SDS-PAGE and blotted onto nitrocellulose membrane. The blot was immunostained with rabbit polyclonal antiserum anti-HPT-1

(rabbit 5, bleed 2, diluted 1:1000) followed by incubation with secondary antibody conjugated to horseradish peroxidase (goat anti rabbit IgG-HRP, Sigma A0545, dil. 1:5000) as outlined previously in the cell studies. The results were analysed by enhanced chemiluminescence detection.

Animal Groups:

1 Antisense only (24 hours)
2 Antisense & C10 5mg (24 hours)
3 Antisense & C10 25 mg (24 hours)
4 C10 25 mg (24 hours)
5 C10 5 mg (48 hours)
6 Antisense & C10 5 mg (48 hours)
7 Antisense & C10 25 mg (48 hours)
8 C10 25 mg (48 hours)
9 Control, untreated

An example of the results obtained is shown in Fig. 3, which shows the expression of rat PT -1 receptor in animals recovered for 24hrs after treatment with antisense oligonucleotide or 25mg C 10 respectively or with a mixture of both. Despite the fact that the level of rat PT-1 expression (120 kDa protein band) is variable from animal to animal within the same treatment group, all four of the rats which were treated with antisense only (i.e. without enhancer) express the protein of interest, whereas only 1 animal treated with the enhancer/antisense combination shows expression of the receptor.

Fig. 4 shows the expression of rat PT-1 receptor in animals recovered for 48hrs after treatment with

antisense oligonucleotide in combination with 5mg or 25mg of C10. In this group of rats treated with the lower concentration of C10, 2 rats out of 3 show expression of the protein of interest while the level of expression for the animals treated with 25mg C10 together with the oligonucleotide S-PTH-03 is decreased in all the rats analysed.

Osmolarity and pH values for each formulation injected are given in Table 1 which also provides the weight of the animals both at the beginning and at the end of the study.

This data presented in Figs. 3 and 4 indicate that co-administration of an enhancer plus the antisense oligonucleotide results in decreased expression in the rat PT-1.

Table 1: Osmolarity and pH values for the formulations injected into rats and animal weights

Animal no	Group no	Duration of study	formulation pH	Osmolarity	Weight day 1	Weight 24 hrs	Weight 48 hrs
1	Group 1	24 Hrs	7.15	318 mOsm	0.194 Kg	0.189 Kg	N/A
2	Group 1	24 Hrs	7.15	318 mOsm	0.215 Kg	0.210 Kg	N/A
3	Group 1	24 Hrs	6.92	292 mOsm	0.194 Kg	0.190 Kg	N/A
4	Group 1	24 Hrs	6.93	291 mOsm	0.190 Kg	0.186 Kg	N/A
5	Group 2	24 Hrs	8.36	459 mOsm	0.214 Kg	0.211 Kg	N/A
6	Group 2	24 Hrs	8.36	468 mOsm	0.195 Kg	0.191 Kg	N/A
7	Group 2	24 Hrs	8.28	472 mOsm	0.196 Kg	0.191 Kg	N/A
8	Group 2	24 Hrs	8.18	469 mOsm	0.216 Kg	0.212 Kg	N/A
9	Group 3	24 Hrs	8.91	615 mOsm	0.192 Kg	0.192 Kg	N/A
10	Group 3	24 Hrs	8.86	610 mOsm	0.202 Kg	0.202 Kg	N/A
11	Group 3	24 Hrs	8.98	558 mOsm	0.236 Kg	0.231 Kg	N/A
12	Group 3	24 Hrs	8.82	562 mOsm	0.230 Kg	0.228 Kg	N/A
13	Group 4	24 Hrs	8.89	598 mOsm	0.190 Kg	0.187 Kg	N/A
14	Group 4	24 Hrs	8.84	597 mOsm	0.210 Kg	0.205 Kg	N/A
15	Group 4	24 Hrs	8.90	577 mOsm	0.210 Kg	0.207 Kg	N/A
16	Group 4	24 Hrs	8.98	579 mOsm	0.210 Kg	0.205 Kg	N/A
17	Group 5	48 Hrs	6.96	293 mOsm	0.223 Kg	0.219 Kg	0.222 Kg
18	Group 5	48 Hrs	6.95	295 mOsm	0.225 Kg	0.221 Kg	0.224 Kg
19	Group 5	48 Hrs	6.72	283 mOsm	0.212 Kg	0.210 Kg	0.213 Kg
20	Group 5	48 Hrs	6.73	286 mOsm	0.200 Kg	0.201 Kg	0.202 Kg
21	Group 6	48 Hrs	8.59	447 mOsm	0.231 Kg	0.227 Kg	0.229 Kg
22	Group 6	48 Hrs	8.24	444 mOsm	0.230 Kg	0.229 Kg	0.230 Kg
23	Group 6	48 Hrs	7.93	444 mOsm	0.200 Kg	N/A	N/A
24	Group 6	48 Hrs	8.00	443 mOsm	0.202 Kg	0.200 Kg	0.205 Kg
25	Group 7	48 Hrs	8.59	577 mOsm	0.203 Kg	0.205 Kg	0.203 Kg
26	Group 7	48 Hrs	8.59	582 mOsm	0.207 Kg	0.203 Kg	0.205 Kg
27	Group 7	48 Hrs	8.98	586 mOsm	0.199 Kg	0.197 Kg	0.207 Kg
28	Group 7	48 Hrs	8.94	549 mOsm	0.227 Kg	0.224 Kg	0.224 Kg
29	Group 8	48 Hrs	8.57	581 mOsm	0.193 Kg	0.191 Kg	0.191 Kg
30	Group 8	48 Hrs	8.55	571 mOsm	0.220 Kg	0.222 Kg	0.219 Kg
31	Group 8	48 Hrs	8.97	595 mOsm	0.216 Kg	0.214 Kg	0.215 Kg
32	Group 8	48 Hrs	8.95	598 mOsm	0.225 Kg	0.223 Kg	0.225 Kg

Example 4 - Effect of co-administration of lipofectamine (cationic lipid) - plasmid DNA (coding for β-galactosidase) complexes with enhancers C8 and C10: Co-administration of the cationic lipid::plasmid DNA complexes increases the transfection of genes into mammalian cells.

A series of transfections examined the effect of the enhancers C8 and C10 on lipofectamine-mediated gene delivery to Caco-2 cells. The DNA used for transfection was a Qiagen maxi-prep of pHM(*lacZ*)6 (0.5μg/μl).

Lipofectamine transfections were accomplished using the following procedure: 3μg (6μl) DNA (solution A) was mixed with 12μl lipofectamine (solution B) [See Table 2 below]. This was a 6X mix. The solution was maintained at room temperature for 15 minutes. The required volume of enhancer stock solution was added [C8 Stock (Na Caprylate): 100mM = 166.2mg in 10ml H_2O; C10 Stock (Na Caprate): 10mM = 19.42mg in 10ml H_2O] followed by addition of Opti-MEM medium up to 600μl. Caco-2 cells grown in the 96-well plate format were washed once with PBS (at 37°C).

The protocol for establishing Caco-2 cells in 96-well tissue-culture plates is as follows: Caco-2 cells were grown in a 30ml 162cm^2 tissue culture flask to 80% confluency. Cells were suspended in 10ml trypsin-EDTA, centrifuged and resuspended in 5ml complete Caco-2 media. Cell number was determined using a haemocytometer where cells were stained with tryptan blue. 1 x 10^5 cells were seeded within each well of the 96 well plate (1 x 10^6 cells were resuspended in **20**ml complete Caco-2 media and

200μl aliquots were added to each well of the 96-well plate). Cells were allowed to grow for 24 hours at 37°C with 5% CO_2 prior to transfection.

100μl of DNA mix was added to each of six different wells in the 96-well plates and transfections were left at 37°C (5% CO_2 for 4 hours. The transfection medium was removed and the cells were washed once with PBS (at 37°C). Fresh complete DMEM (100μl) was added to the cells and left O/N at 37°C in the incubator. Cells were assayed for β-galactosidase activity 24 hours post transfection.

Table 2

Trans	Solution A	Solution B	Enhancer vol.
1	6μl DNA + 54μl OptiMEM	12μl Lipofectamine + 48μl OptiMEM	-
2	6μl DNA + 54μl OptiMEM	12μl Lipofectamine + 48μl OptiMEM	180 μl C8 (30mM)
3	6μl DNA + 54μl OptiMEM	12μl Lipofectamine + 48μl OptiMEM	150 μl C10 (5mM)
4	6μl DNA + 54μl OptiMEM	12μl Lipofectamine + 48μl OptiMEM	30 μl C8 (5mM)
5	6μl DNA + 54μl OptiMEM	12μl Lipofectamine + 48μl OptiMEM	25 μl C10 (1mM)

Fig. 5 demonstrates that the penetration enhancers C8 and C10 also facilitate the intracellular delivery of plasmid DNAs into Caco-2 cells when the plasmid DNA is complexed with a model cationic lipid such as lipofectamine. In these studies:

Enhancer system 1A: = 30mM C8

Enhancer system 2A: = 5mM C10

Enhancer system 1B: = 5mM C8

Enhancer system 2B: = 1mM C10

Setting the efficiency of DNA transfection with the cationic lipid: plasmid DNA complex at 100%, in the presence of the enhancers C8 and C10 the efficiency of transfection increases to 143% and 245%, respectively. This demonstration and discovery has general, broad application for the intracellular delivery of plasmid DNAs and genes to mammalian cells using non-viral vector systems such as, by way of example but without limitation, cationic lipids, PEI (polyethyleneimine) systems, polyanhydride systems, chitosan systems, cellulose systems, dendrimeric based systems, liposomes, PLGA DNA particles co-administered with enhancers such as C8 and C10 and systems involving coadministration of a nucleic acid-based drug in conduction with an enhancer.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications fall within the scope of the invention.

CLAIMS

1. A method for enhancing the intracellular delivery of a nucleic acid-based drug in a mammal comprising administering to the mammal, in combination with the nucleic acid-based drug, an enhancer in an amount effective to enhance the intracellular delivery of the nucleic acid-based drug.

2. An *in vitro* method for enhancing the intracellular delivery of a nucleic acid-based drug in a mammalian cell or tissue sample comprising contacting the cells or tissue with the nucleic acid-based drug and contacting the cells or tissue with an enhancer in an amount effective to enhance the intracellular delivery of the nucleic acid-based drug.

3. The method of claim 1 or claim 2, wherein the enhancer is a fatty acid or an ester, ether, salt or derivative thereof.

4. The method of claim 3 wherein the enhancer has a carbon chain length of from 4 to 24 carbon atoms, with the provisos that (i) where the enhancer is an ester of a fatty acid, said chain length relates to the chain length of the carboxylate moiety, and (ii) where the enhancer is an ether of a fatty acid, at least one alkoxy group has a carbon chain length of from 4 to 24 carbon atoms.

5. The method of claim 3 wherein the enhancer is a medium or long chain fatty acid or an ester, ether, salt or derivative thereof.

6. The method of claim 5 wherein the enhancer is a medium chain fatty acid or an ester, ether, salt or derivative thereof.

7. The method of claim 6 wherein the enhancer has a carbon chain length of from 8 to 14 carbon atoms, with the provisos that (i) where the enhancer is an ester of a fatty acid, said chain length relates to the chain length of the carboxylate moiety, and (ii) where the enhancer is an ether of a fatty acid, at least one alkoxy group has a carbon chain length of from 8 to 14 carbon atoms.

8. The method of claim 7 wherein the enhancer is caprylic acid, nonanoic acid, capric acid, undecanoic acid, lauric acid or an ester, ether, salt or derivative thereof.

9. The method of claim 8 wherein the enhancer is caprylic acid, capric acid or an ester, ether, salt or derivative thereof.

10. The method of claim 9 wherein the enhancer is caprylic acid or an ester, ether, salt or derivative thereof.

11. The method of any preceding claim wherein the enhancer is a sodium salt of a fatty acid.

12. The method of claim 1, wherein the enhancer is selected from the group consisting of babassu oil, a fish oil, medium chain glyceride mixes and an acylcarnitine.

13. The method of any preceding claim for enhancing the cytoplasmic and/or nuclear delivery of the nucleic acid-based drug.

14. The method of any preceding claim wherein the cell or tissue sample is epithelial.

15. The method of any preceding claim wherein the cell or tissue sample is from the gastrointestinal tract.

16. The method of claim 15 wherein the cell or tissue sample is of the small intestine.

17. The method of any preceding claim, wherein the nucleic acid-based drug is selected from the group consisting of an oligonucleotide, an antisense oligonucleotide, a plasmid DNA, a gene, a ribozyme, a gene-correcting oligonucleotide, a triple-helix forming oligonucleotide, and an oligonucleotide which functions as adjuvant.

18. The method of claim 17, wherein the oligonucleotide is selected from the group consisting of an oligonucleotide having a modified backbone chemistry, an oligonucleotide having a modified sugar or terminal group, a chimeric oligonucleotide comprised of nucleotides of different chemistries, and an oligonucleotide having MOE chemistry.

19. The method of claim 17, wherein the gene is selected from a gene coding for a protein, a gene coding for an RNA molecule which functions in an antisense

capacity when expressed within mammalian cells and a gene coding for a ribozyme.

20. The method of any preceding claim, wherein the amount of enhancer effective to enhance the intracellular delivery is about 0.01mM to 1M.

21. The method of claim 20, wherein the amount of enhancer effective to enhance the intracellular delivery is about 1mM to 100mM.

22. The method of any preceding claim, wherein the molar ratio of the enhancer to the nucleic acid-based drug is 1:100 to 100:1.

23. The method of any preceding claim, wherein the enhancer and nucleic acid-based drug are administered or contacted at the same time.

24. The method of any one of claims 1 to 22, wherein the enhancer and nucleic acid-based drug are administered or contacted sequentially such that the enhancer is administered or contacted first.

25. The method of any one of claims 1 to 22, wherein the enhancer and nucleic acid-based drug are administered or contacted sequentially such that the nucleic acid-based drug is administered or contacted first.

26. The method of claim 1, or of any one of claims 3 to 25 as dependent from claim 1, wherein the administration is oral.

27. The method of any preceding claim wherein the nucleic acid-based drug is complexed with a cationic lipid.

28. The method of any one of claims 1 to 26 wherein the nucleic acid-based drug is complexed with a polymer system.

29. The method of any preceding claim wherein the nucleic acid-based drug is entrapped in a polymer system.

30. The method of claim 28 or claim 29, wherein the polymer system is selected from the group consisting of a polyethyleneimine system, a polyanhydride system, a chitosan system, a cellulose system, a dendrimeric based system, and PLGA particles.

31. Use of an enhancer in the preparation of a medicament containing a nucleic acid-based drug, for the enhancement of intracellular delivery of said nucleic-acid-based drug.

32. A pharmaceutical composition for enhancing the intracellular delivery of a nucleic acid-based drug comprising (1) a pharmaceutically effective amount of a nucleic acid-based drug and (2) an enhancer in an amount effective to enhance the intracellular delivery of the nucleic acid-based drug.

33. The use of claim 31, or the composition of claim 32, wherein the enhancer is as defined in any one of claims 2 to 12.

34. The use or composition of any one of claims 31 to 33 wherein the drug is as defined in any one of claims 17 to 19 or 27 to 30.

35. The use or composition of any one of claims 31 to 34 wherein the enhancer is used or is present in an amount sufficient to enhance cytoplasmic and/or nuclear delivery of the nucleic acid-based drug.

36. The use or composition of any one of claims 31 to 35 wherein the cell is as defined in any one of claims 14 to 16.

37. The use or composition of any one of claims 31 to 36 wherein the amount of the enhancer, or the ratio of enhancer to drug, is as defined in any one of claims 20 to 22.

38. The method, use or composition of any preceding claim wherein an inhibitor of an enzyme that degrades the nucleic acid-based drug or which transports a nucleic acid-based drug back out of the cell is also administered, brought into contact with the cell or tissue sample, contained in the medicament, or present in the composition.

39. The method, use or composition of claim 38 wherein the inhibitor is a P-glycoprotein inhibitor.

40. The method, use or composition of any preceding claim wherein an endosome escape/nuclear accumulation agent is also administered, brought into contact with the cell or tissue sample,

contained in the medicament, or present in the composition.

41. The method, use or composition of any preceding claim wherein the nucleic acid-based drug is condensed by a DNA condensing agent.

42. The method, use or composition of any preceding claim wherein condensed DNA is complexed with cationic lipid and is co-administered with the enhancer, brought into contact with the cell or tissue sample simulataneously with the enhancer, or contained in the medicament or present in the composition along with the enhancer.

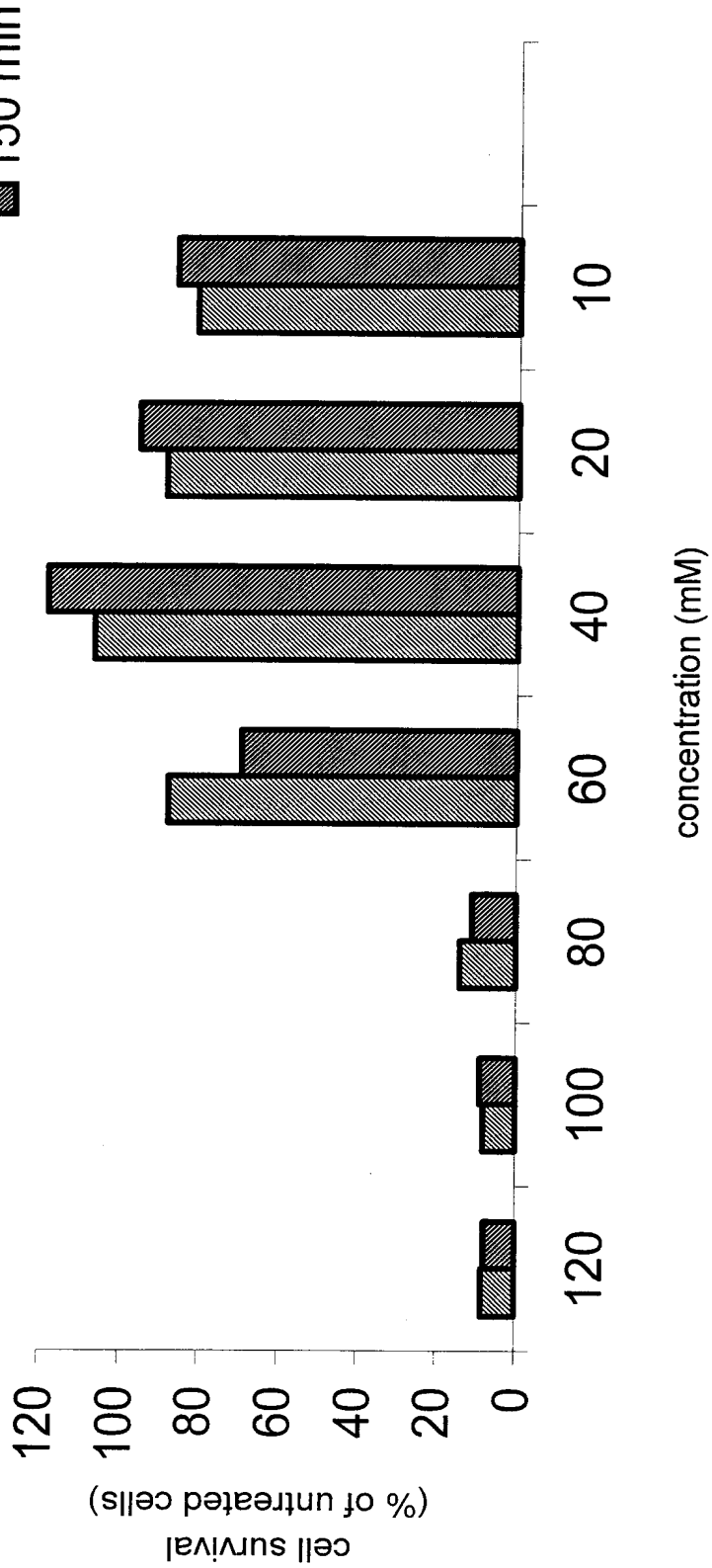

30 min
150 min
cell survival
(% of untreated cells)
0
20
40
60
80
100
120
concentration (mM)
10
20
40
60
80
100
120

FIG 1a

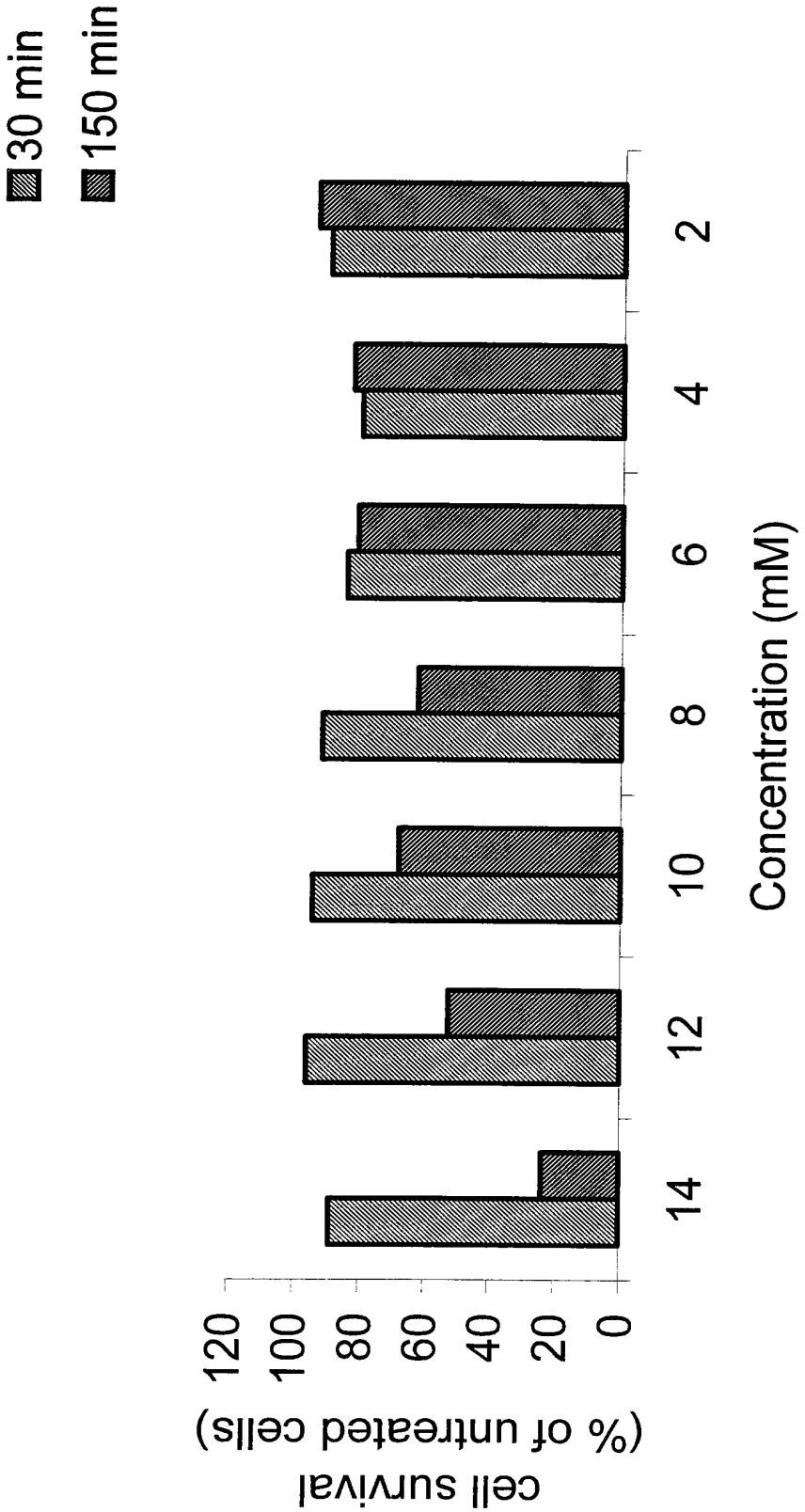

30 min
150 min
cell survival
(% of untreated cells)
120
100
80
60
40
20
0
2
4
6
8
10
12
14
Concentration (mM)

FIG 1b

FIG 2

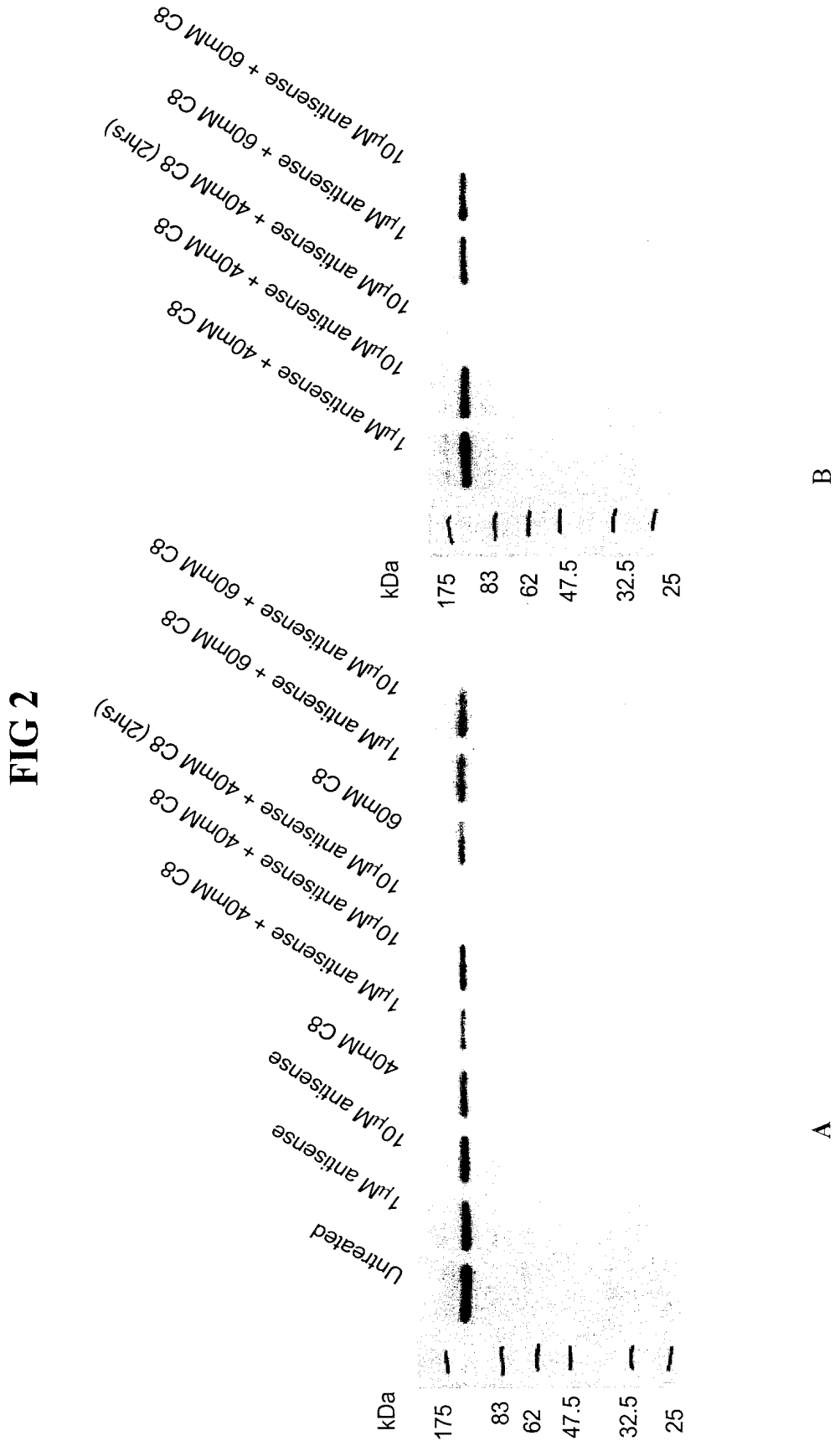

A
kDa
175
83
62
47.5
32.5
25
Untreated
1μM antisense
10μM antisense
40mM C8
1μM antisense + 40mM C8
10μM antisense + 40mM C8
60mM C8
1μM antisense + 40mM C8 (2hrs)
10μM antisense + 40mM C8 (2hrs)
1μM antisense + 60mM C8
10μM antisense + 60mM C8
B
kDa
175
83
62
47.5
32.5
25
1μM antisense + 40mM C8
10μM antisense + 40mM C8
1μM antisense + 40mM C8 (2hrs)
10μM antisense + 40mM C8 (2hrs)
1μM antisense + 60mM C8
10μM antisense + 60mM C8

FIG 3

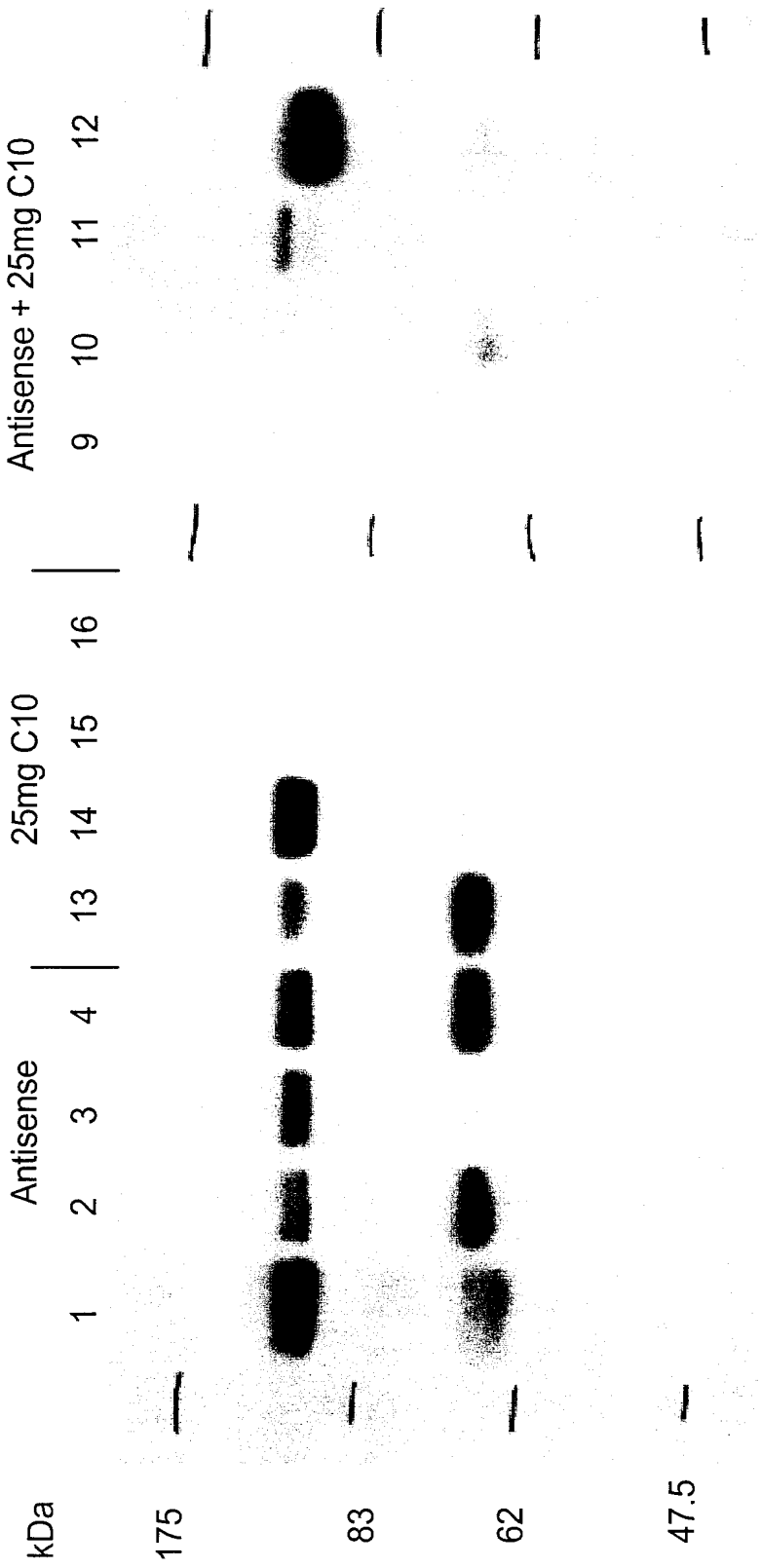

kDa
175
83
62
47.5
Antisense
1
2
3
4
25mg C10
13
14
15
16
Antisense + 25mg C10
9
10
11
12

FIG 4

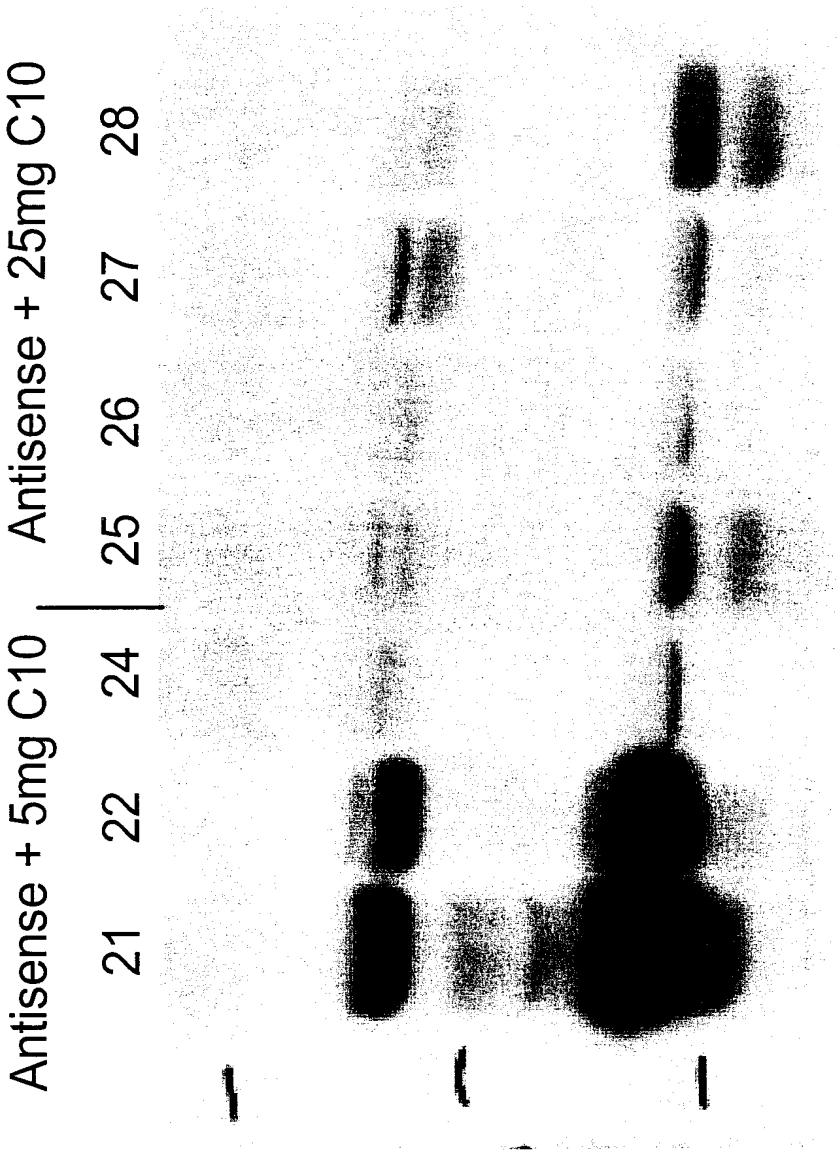
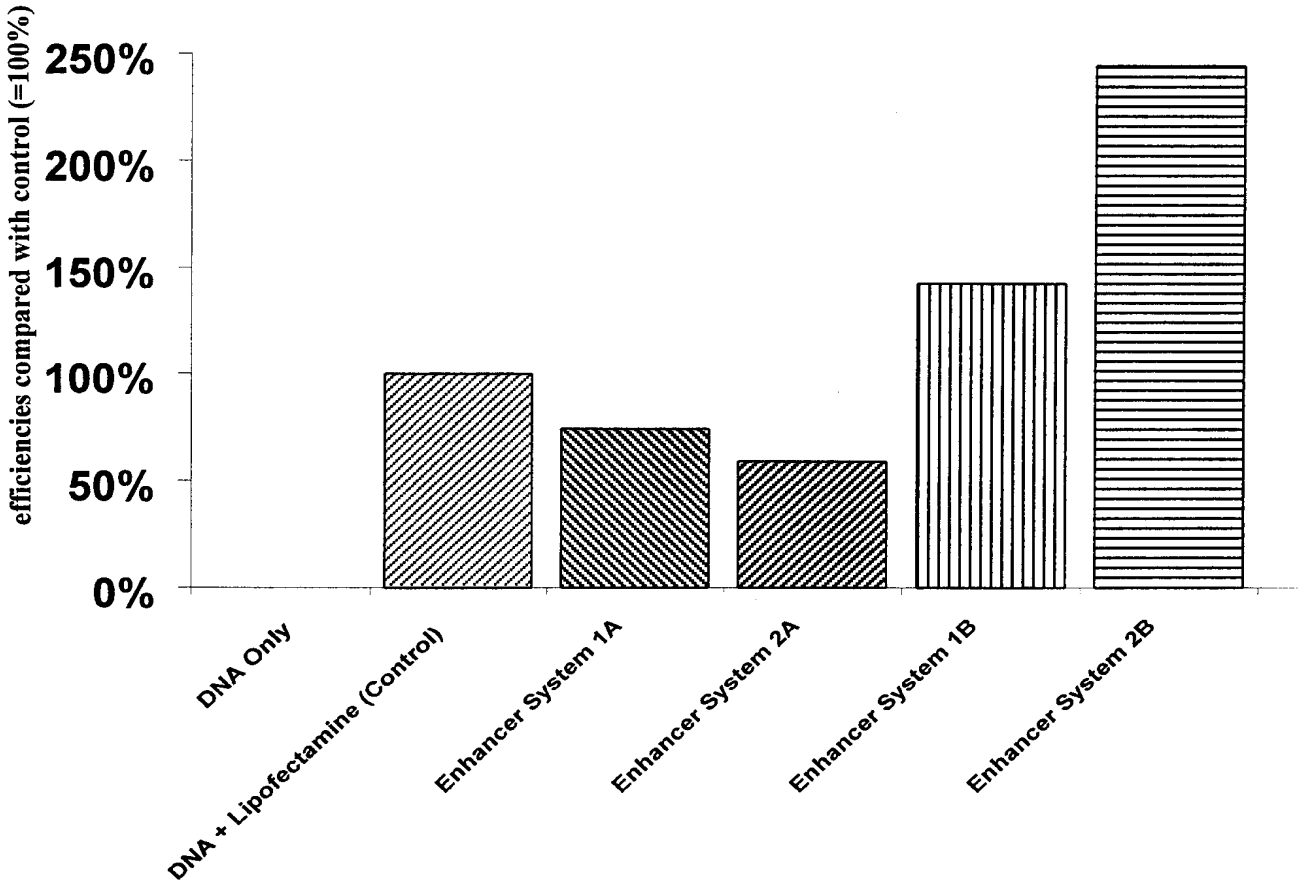


FIG 5


efficiencies compared with control (=100%)
250%
200%
150%
100%
50%
0%
DNA Only
DNA + Lipofectamine (Control)
Enhancer System 1A
Enhancer System 2A
Enhancer System 1B
Enhancer System 2B